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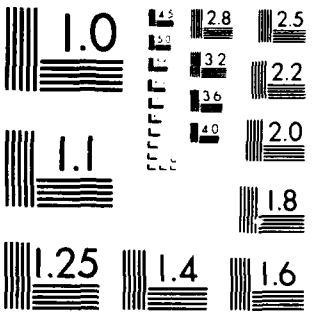

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BIOLOGICAL SYNTHESIS OF A PROTEIN ANALOGUE OF ACETYLCHOLINESTERASE: MONOCLONAL ANTI-IDIOTYPE ANTIBODY ANALOGUE OF THE ESTERATIC SITE

ANNUAL REPORT

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JULY 10, 1986

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We have studied the binding properties and inhibitory activity of AE-2, a monoclonal antibody which binds to (AChE) and inhibits its activity by up to 50%. This antibody was compared to C1B7, another inhibitory enzyme. It was determined that these antibodies bind to different sites on AChE; these sites were distinct from the active site and from the binding sites for a number of anticholinergic drugs, including DFP, propidium, endrophonium and neostigmine. A kinetic analysis of inhibition by AE-2 and C1B7 suggested that their inhibition of AChE was non-competitive and allosteric.

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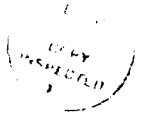
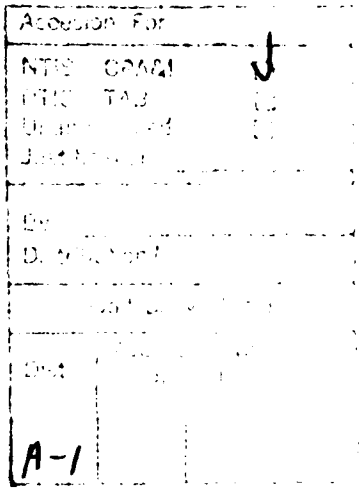
FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the porducts or services of these organizations.





REPORT

Problem:

The goals of this research are to biochemically and immunochemically characterize the molecular structure of the active site of human acetylcholinesterase (AChE). Our research during the third year has been directed toward characterizing monoclonal antibodies that are capable of inhibiting the catalytic activity of AChE.

Background:

AChE is associated chiefly with cells involved in cholinergic synaptic transmission and is also found in a few non-neuronal cells like erythrocytes. The enzyme exists in a variety of aggregation states with a monomer unit of about 75,000 Mr (Biochem. Biophys. Acta 236, 612-618, 1971; Biochem. J. 133, 521-527, 1973; Eur. J. Biochem 88, 119-125, 1978).

All of the molecular species of AChE appear to have the same enzymatic sites. Fambrough et al. (Proc. Natl. Acad. Sci. USA 79, 1078-1082, 1982) prepared five monoclonal antibodies that bound to purified human erythrocyte AChE, each reacting with different antigenic sites on the AChE molecule. All of these antibodies cross-reacted with human and monkey neuromuscular junctions. It was concluded that a high degree of homology exists between the AChE of erythrocytes and neuromuscular junctions.

We have now identified and characterized the interaction of several monoclonal antibodies with human erythrocyte AChE, and we have demonstrated that two such antibodies, C1B7 and AE-2, inhibit the activity of the enzyme by binding to novel allosteric sites on the molecule.

Studies using Monoclonal Antibodies:

I. Inhibitory Monoclonal Anti-AChE Antibodies

Sixteen mouse monoclonal antibodies that bind to human red blood cell AChE were produced in four separate fusions. Most of the antibodies as well as five others (AE-1, AE-2, AE-3, AE-4, and AE-5) previously described by Fambrough et al. were of the IgG,k class with Kd values for binding to AChE in the range of 9 to 200 nM (Table 1). All of the antibodies were tested for their ability to inhibit AChE activity. Only two of the 21 antibodies, C1B7 and AE-2, significantly inhibit enzyme activity (Fig. 1). C1B7 was found to inhibit AChE activity by as much as 85-90% at relatively low concentrations. In contrast, higher concentrations of AE-2 were required to achieve maximum inhibition at about 75%. The two inhibitory antibodies had intermediate Kd values: 20 nM for C1B7 and 50 nM for AE-2.

II. Distinct Binding Sites for C1B7 and AE-2 on AChE

In order to determine whether C1B7 and AE-2 bound to similar or different regions of the AChE molecule, competition assays were performed in which ^{125}I-labeled AChE was preincubated with one of the inhibitory antibodies and then tested for subsequent binding to the second inhibitory antibody. These experiments, summarized in Table 2, demonstrate that C1B7 and AE-2 antibodies bind to different sites on AChE. Preincubation of labeled AChE with AE-2 had no effect on subsequent binding of AChE to C1B7

but almost completely blocked subsequent binding to AE-2. Similarly, preincubation of AChE with C1B7 did not inhibit subsequent binding to AE-2 but did block binding of AChE to C1B7. Among the non-inhibitory antibodies tested, only AE-1 showed any effect. Preincubation with C1B7 or AE-1 inhibited subsequent binding of AChE to the other. AE-1 did not, however, significantly affect AE-2 binding.

TABLE 1

ANTI-ACETYLCHOLINESTERASE ANTIBODIES

Isotypes and binding constants of 16 monoclonal antibodies prepared from four separate fusions in this laboratory, and of antibodies AE-1, -2, -3, -4, and -5 which have been previously reported by Fambrough et al, PNAS 79, 1078, 1982.

Ab Source	Ab Name	Isotype[a]	Ka[b]
		nM	10^7 M^{-1}
Fusion 1	SA5	IgM,k	2.5
Fusion 2	F3F2	IgM,k	1.7
	F3G10	IgM,k	1.1
Fusion 3	C1B7	IgG1,k	5.0
	C1C7	IgG1,k	ND
	C1C8	IgG1,k	1.8
	C1D7	IgG1,k	0.7
	C1F9	IgG1,k	ND
	C1G4	IgG1,k	0.8
	C1H7	IgG1,k	ND
	C1H9	IgG1,k	11.4
	C1H11	IgG1,k	ND
Fusion 4	C2A7	IgG1,k	ND
	C2B8	IgG1,k	ND
	C2C4	IgG1,k	0.5
	C2H7	IgG1,k	6.9
Fambrough	AE-1	IgG1,k	1.0
	AE-2	IgG1,k	2.0
	AE-3	IgG1,k	20.0
	AE-4	IgG1,k	6.0
	AE-5	IgG1,k	15.0

[a] Isotypes were determined using a Zymed immunoassay kit.
[b] Binding constants were calculated from Scatchard plots of antibody binding to AChE

TABLE 2

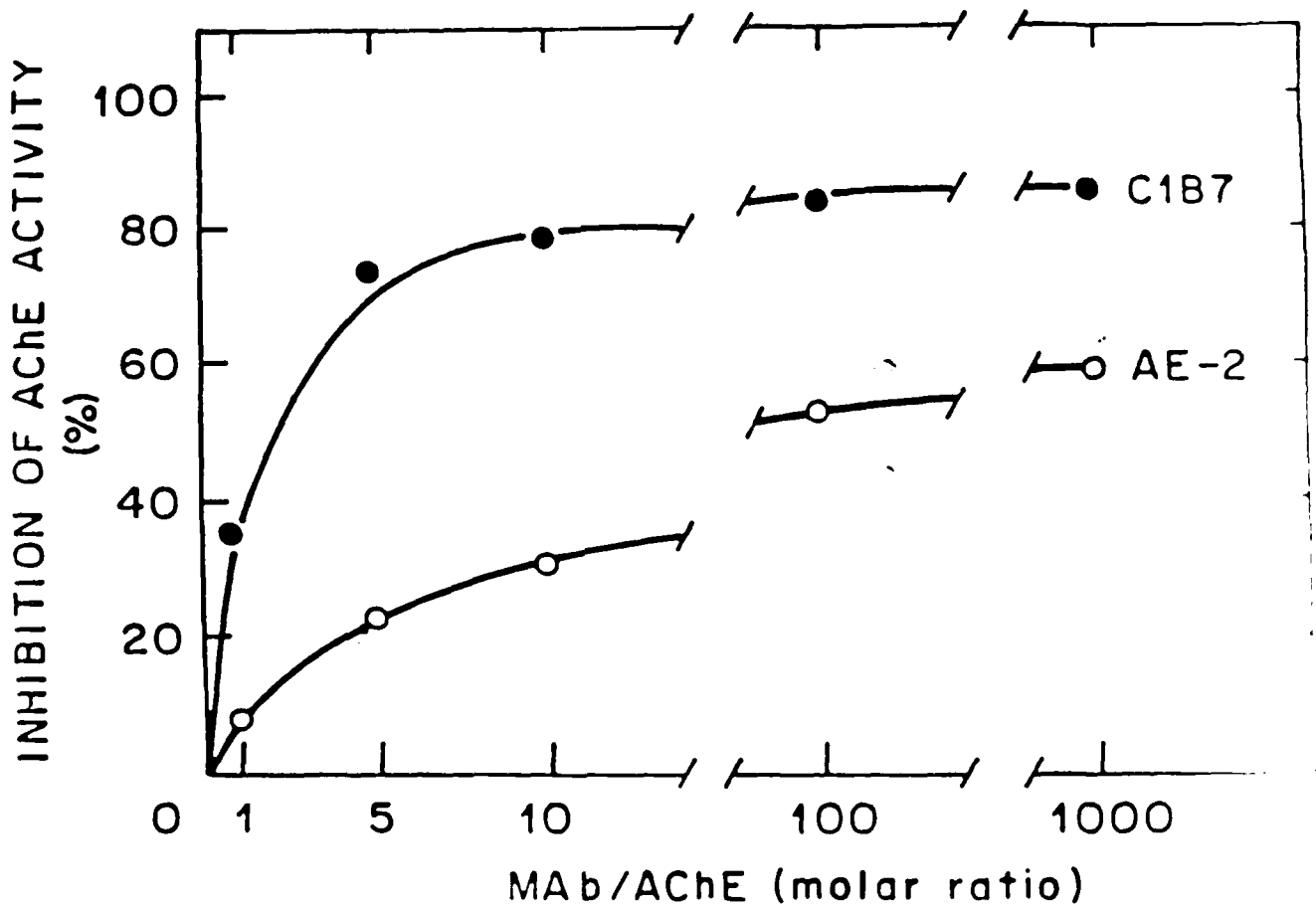
COMPETITION OF ANTIBODIES FOR BINDING TO AChE

Immobilized[a]	Inhibition of ^{125}I-AChE Binding by Competing Antibody (%)[b]		
	AE-2	C1B7	AE-1
C1B7	4.4	89.9	97.7
AE-2	88.7	0.0	8.7
AE-1	12.8	66.7	97.9

[a] A 100-fold molar excess of one monoclonal antibody was preincubated with 0.26 pmoles ^{125}I-AChE in 0.5 ml PBS for 30 min at 4° C, and 50 ul of this mixture were added to wells containing antibody immobilized on goat anti-mouse IgG.

[b] Control binding: C1B7 19,500 cpm
AE-2 11,500 cpm
AE-1 19,500 cpm

FIGURE 1


INHIBITION OF AChE ACTIVITY (%)
100
80
60
40
20
0
1
5
10
100
1000
MAb/AChE (molar ratio)
C1B7
AE-2

III. Inhibition of Monomeric and Dimeric AChE by C1B7 and AE-2

Since erythrocyte AChE is composed of two subunits, it was of interest to determine whether the antibodies bind to and inhibit activity of the individual subunits as well as to the intact dimer. Conditions for mild reduction and alkylation were established that resulted in complete dissociation of the AChE subunits with retention of >90% of the enzymatic activity. Subunit dissociation was verified by SDS-PAGE analysis of the enzyme. Following reductive alkylation AChE migrated as a single ~75 kDa band under both reducing and non-reducing conditions, indicating complete reduction of the disulfide linkage and formation of monomers. When these fully active AChE monomers were treated with C1B7 or AE-2, for 30 min at 25°C, inhibition was essentially identical to that observed for the native AChE dimer (73.9% inhibition for monomeric vs. 79.8% for dimeric C1B7; 55.2% inhibition for monomeric vs. 58.0% for dimeric AE-2).

Two mechanisms are possible to account for the finding that AE-2 inhibits enzyme activity only up to 75%. (1) AE-2 recognizes and binds only to half or more of the monomers and inhibits these totally; or (2) AE-2 binds all of the monomers and only partially inhibits the activity of each. The results of two further experiments indicated that the antibody binds both monomers. First, AChE monomers and dimers were quantitatively absorbed by AE-2 bound to Sepharose beads, as determined by monitoring the disappearance of enzyme activity. Second, when binding of AChE monomers and dimers was monitored by directly assaying enzyme activity bound to AE-2 immobilized on microtiter plates, weight-equivalent activity was detected.

IV. Distinct Binding Sites for Anticholinesterase Compounds and Inhibitory Antibodies on AChE

The possibility that C1B7 and AE-2 inhibit enzyme activity by binding directly to the active site or to previously identified allosteric sites was investigated. Competition assays in which AChE was pretreated with well-defined anticholinesterase compounds revealed no specific inhibitory effect on antibody binding to the pretreated AChE. The following anticholinesterase compounds were tested: the irreversible esteratic site-directed inhibitor DFP, the reversible site-directed inhibitors neostigmine, carbachol, edrophonium, and BW284c51; and the allosteric site-directed inhibitors gallamine and propidium (Table 3). Pharmacologic levels (i.e., concentrations of drug that are just sufficient to inhibit enzyme activity completely) had no effect at all on antibody binding. In subsequent experiments, much higher levels (at least 1000-fold greater than the Kd) of each drug were also tested for inhibition of antibody binding, and no specific inhibition of antibody binding was observed. That is, 10-100 mM levels of the various anticholinesterase drugs prevented the binding of non-inhibitory antibodies (i.e. AE-1 and C1B7) in addition to the binding of the inhibitory antibodies. Acetylthiocholine, which is used as the substrate for AChE in the Ellman assay, also had no effect on C1B7 or AE-2 binding to AChE in concentrations up to 20 mM.

Inhibition experiments with the active center sub-site-directed drugs DFP and edrophonium were performed in two different ways. First, enzyme was totally inactivated by preincubation with various levels of DFP or edrophonium. Both the pretreated and untreated enzyme were found to bind to AE-2 and C1B7 equally well. In a second series of experiments, AChE was preincubated with AE-2 or AE-4 and then treated with drug. In this case the kinetics of drug inactivation of residual AChE activity was unaffected by pretreatment with antibody. Thus, the binding of drug to enzyme did not affect antibody binding, nor did antibody binding to enzyme affect drug action on residual AChE activity.

Table 3

ANTICHOLINESTERASE COMPOUNDS COMPETED AGAINST INHIBITORY ANTIBODIES FOR BINDING TO AChE

Drug	AChE Site	Kd of Drug for AChE[a]	Concentration Tested in Binding Assay[b]
		M	M
DFP	Esteratic	10^{-6}	10^{-7} - 10^{-2}
Neostigmine	Esteratic Anionic P1	10^{-7}	10^{-6} - 10^{-5}
Carbachol	Esteratic Anionic P1	10^{-4}	10^{-3} - 10^{-1}
Edrophonium	Anionic P1	10^{-5} 10^{-4}	10^{-3} - 10^{-1}
BW	Anionic P1	10^{-6}	10^{-3} - 10^{-2}
Gallamine	Anionic P2	10^{-5}	10^{-12} - 10^{-2}
Propidium	P1	10^{-5}	10^{-12} - 10^{-2}

[a] Kd represents the concentration of drug at half-maximal inhibition (esteratic or anionic sites) or dissociation of propidium (peripheral sites), as determined in Ellman assays.

[b] For the binding assay, 0.25 pmoles ^{125}I-AChE in 0.5 ml PBS were preincubated with varying concentrations of drug for 30 min at 25° C, and 50 ul of this mixture were then added to wells containing immobilized antibody.

V. Kinetic Analysis of AChE Inhibition

Since binding of the antibodies was not blocked by site-specific ligands, inhibition must be considered allosteric. In order to analyze the mechanism of allosteric inhibition, assays of enzyme activity were performed at varying concentrations of antibody and substrate. Kinetic parameters characterizing antibody inhibition were derived from Eadie-Hofstee (Fig. 2) and Webb plots (Fig. 3) as described by Webb (Enzyme and Metabolic Inhibitors, 1, Acad. Press, 1963). The data are summarized in Table 4.

V_m fell as antibody concentration increased; it was reduced by 50% ($V_{im}/V_m = 0.50$) at an antibody concentration, $(I_t)_{50}$, of slightly >1 (molar ratio of antibody enzyme) for C1B7 and about 10 for AE-2. In contrast, K_m changed very little as substrate concentration increased: nearly parallel lines were observed in the Eadie-Hofstee plots, and the ratio of their slopes remained close to 1. Thus, the affinity of substrate for the enzyme was unaffected.

It should also be noted that inhibition by either antibody was not total. This effect was measured in the Webb plot, where residual activity (ß) in the presence of an infinite concentration of antibody is estimated from the ordinate intercept (1/1-ß). Using the values of and ß obtained in these plots, reasonable estimates of K_i were obtained.

In order to confirm the non-competitive nature of inhibition by C1B7 and AE-2, and to compare it to the known allosteric nature of substrate inhibition, kinetic assays were performed at very high and at supraoptimal substrate concentrations (Fig. 4). In this case, activity-substrate plots (V vs. S) were chosen as most illustrative of the patterns which result. For both C1B7 and AE-2, little if any change in K_m was observed at low antibody: enzyme ratios. At ratios above 10, shifts in K_m (i.e., substrate concentration at Vim/2 for a particular curve) were observed. The inhibition produced by C1B7 and AE-2, however, could not be fully reversed by increasing the substrate level, even through the optimal substrate concentration was shifted at least three-fold, from 1.0 to 3.0 mM, in the presence of high antibody concentration.

The data in Fig. 4 also demonstrate typical substrate inhibition of AChE activity at mM concentrations. This substrate inhibition was also observed in the presence of antibody. Substrate inhibition and antibody inhibition exhibited summation, i.e., they were found to be essentially additive.

VI. Summation of Inhibition of AChE Activity by C1B7 and AE-2

As shown in Fig. 5, the inhibitory effects of the two antibodies were also found to be additive. In these experiments, AChE was treated sequentially with the two antibodies and AChE activity was subsequently determined. When AChE was pretreated with AE-2, subsequent addition of varying amounts of C1B7 resulted in increased levels of inhibition that were similar to the theoretical sum of the levels of inhibition for the two antibodies acting separately. Similarly, when enzyme was pretreated with C1B7, subsequent treatment with AE-2 resulted in inhibition that was also essentially additive.

FIGURE 2

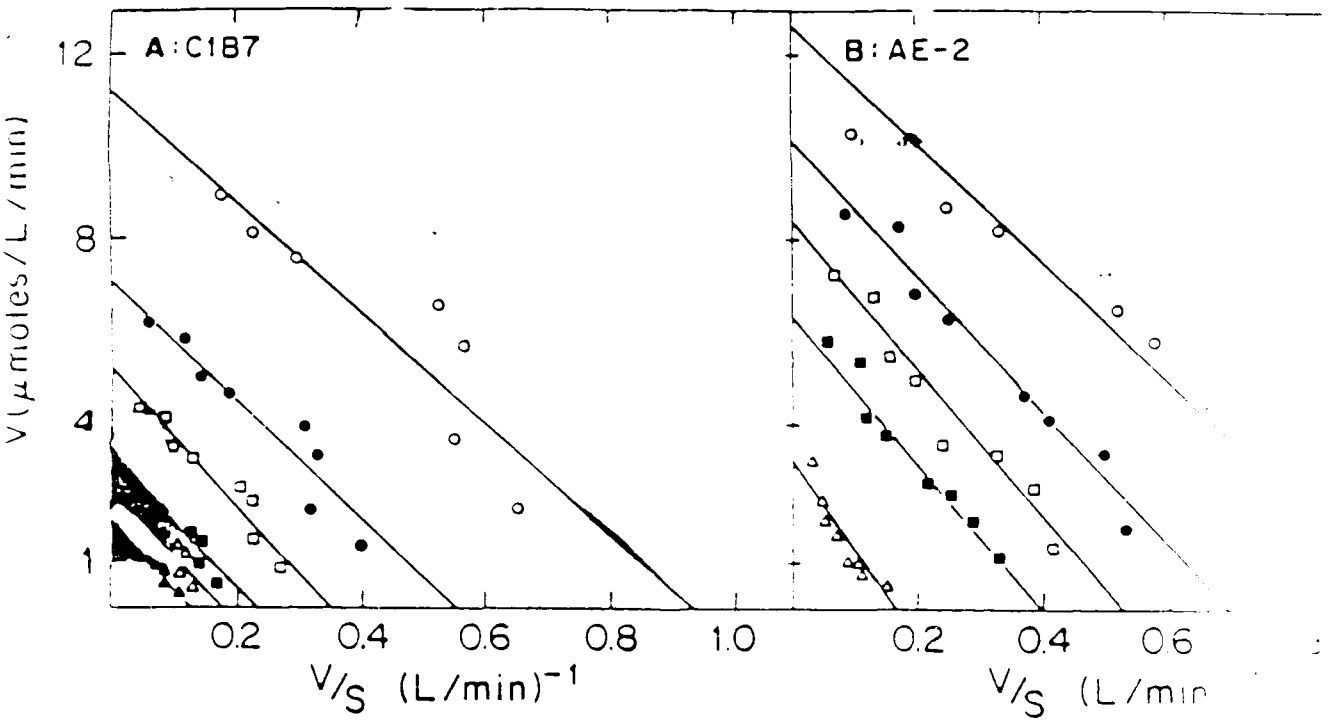


FIGURE 3

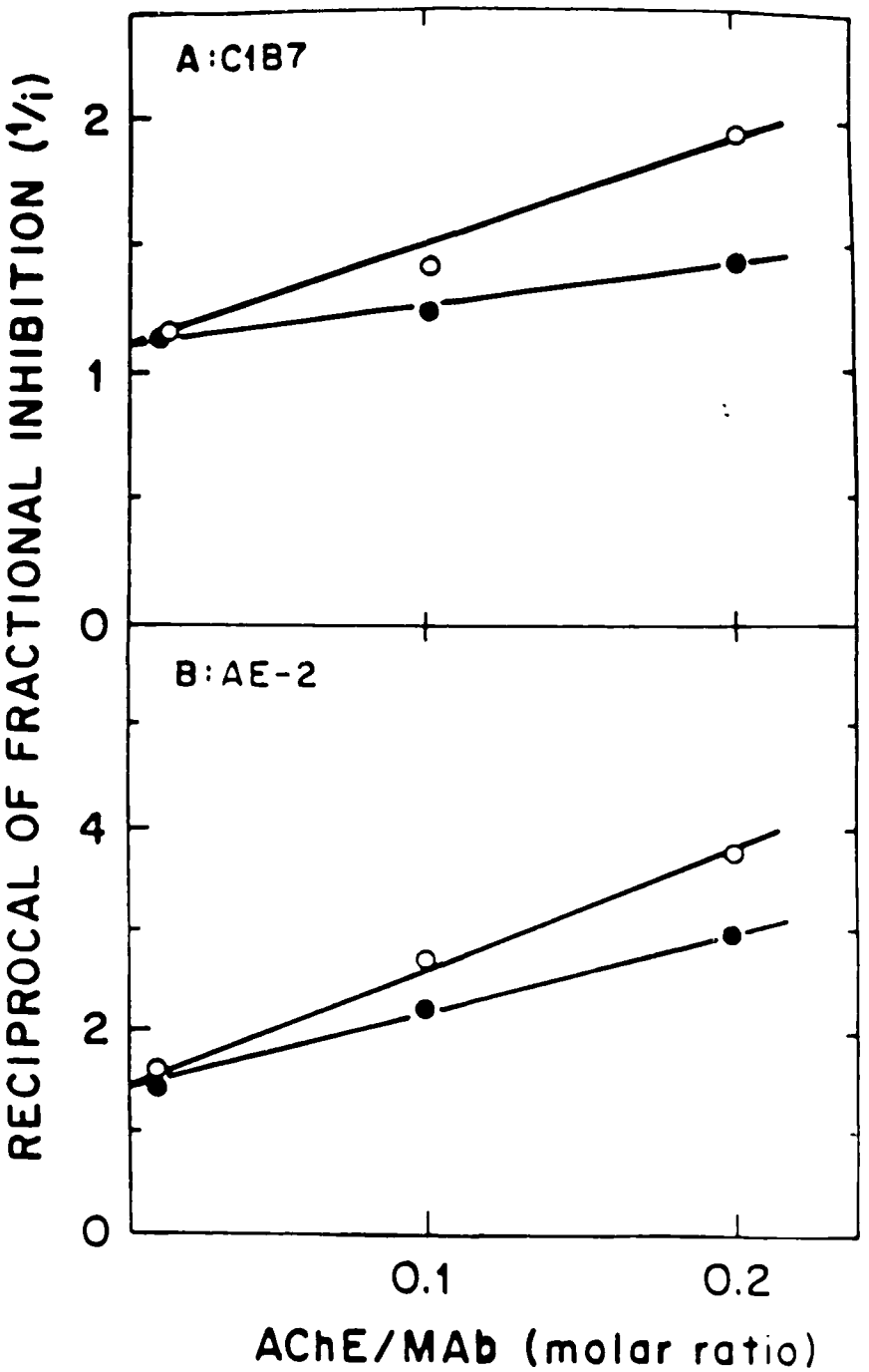

A:C1B7
B:AE-2
RECIPROCAL OF FRACTIONAL INHIBITION (1/i)
0
1
2
0
2
4
0.1
0.2
AChE/MAb (molar ratio)

FIGURE 4

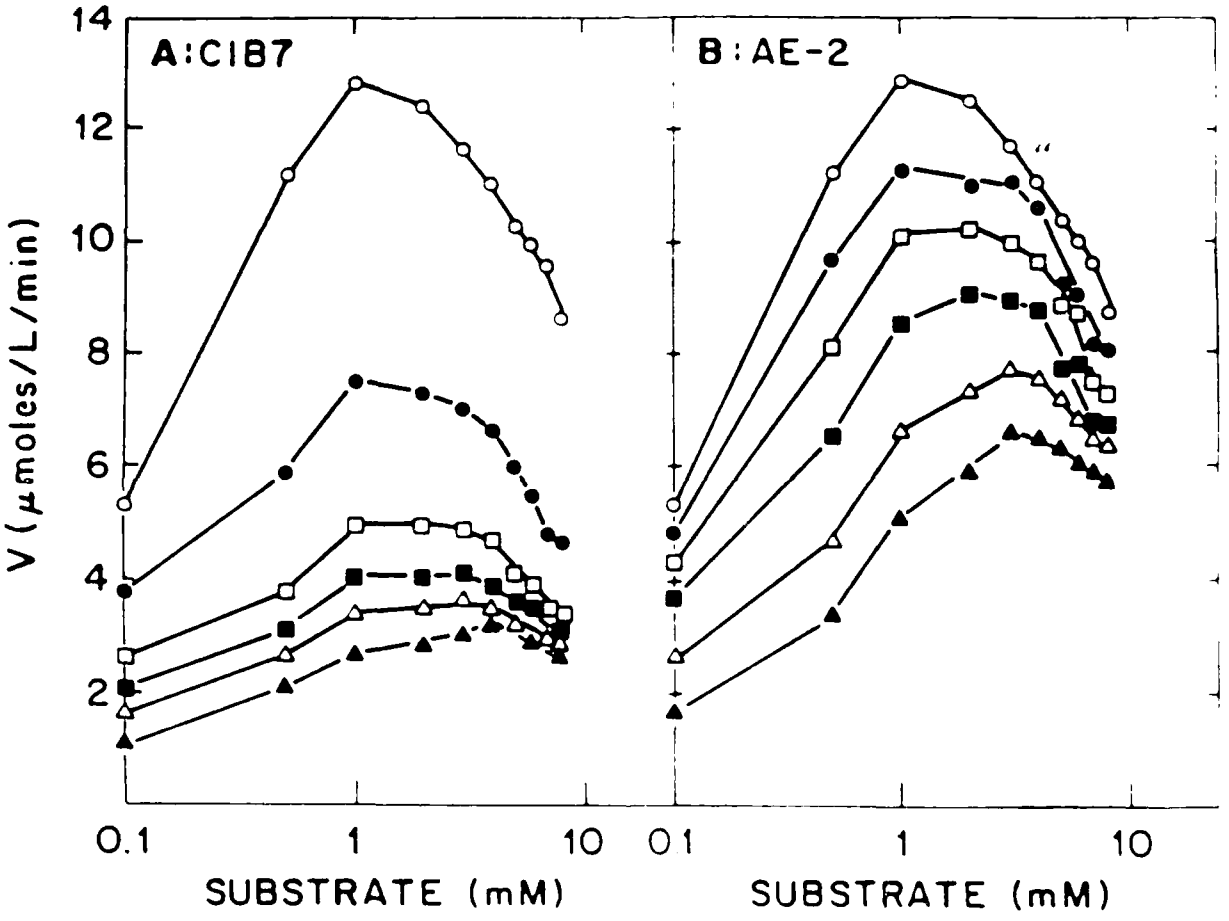

A:CIB7
B:AE-2
V (μmoles/L/min)
14
12
10
8
6
4
2
0.1
1
10
SUBSTRATE (mM)
0.1
1
10
SUBSTRATE (mM)

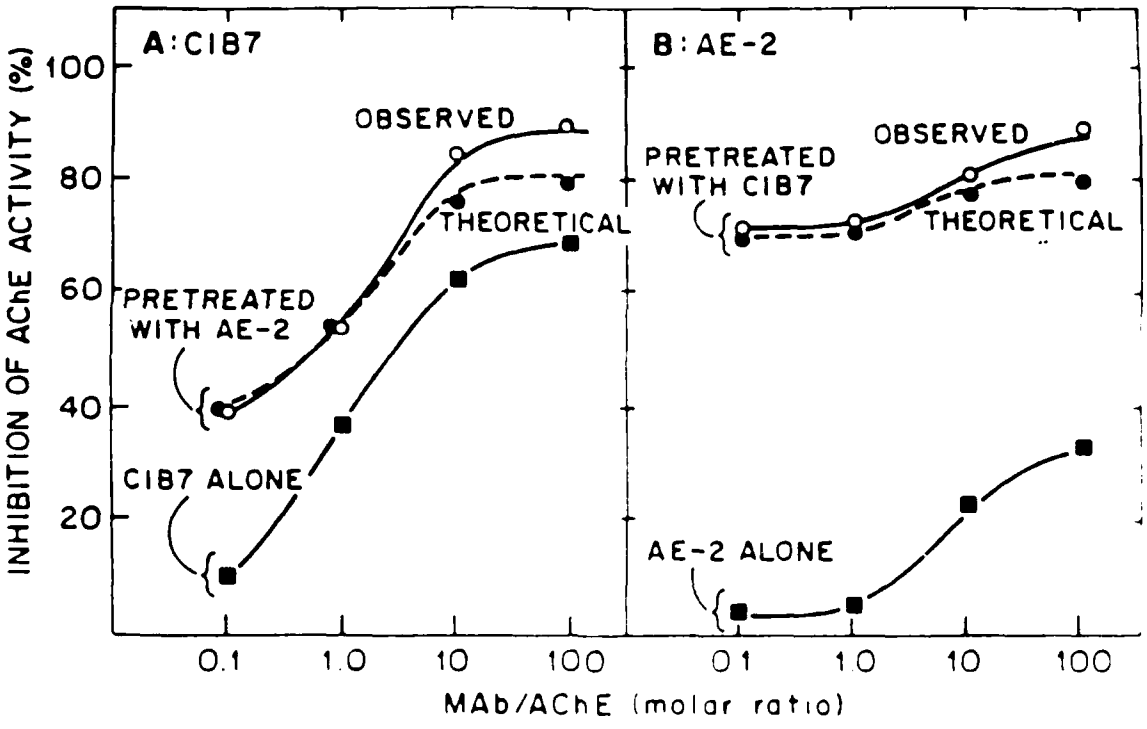
Table 4

KINETIC PARAMETERS OF INHIBITORY ANTIBODIES[a]

Inhibitor		Kinetic Parameter					
MAb	Concentration	V_m	V_{im}/V_m	K_m			K_i
	MAb/AChE	u moles L/min		mM			MAb/AChE
C1B7	0	11.2		0.11			
	1	6.9	0.62	0.12	1.0		0.8
	2	5.2	0.47	0.15	1.3		1.0
	5	3.1	0.28	0.13	1.2		1.1
	10	2.5	0.22	0.13	1.2		1.5
	100	1.8	0.16	0.13	1.2	0.09	8.2
AE-2	0	12.7		0.12			
	2	10.3	0.81	0.15	1.2		5.0
	5	8.4	0.66	0.16	1.3		5.3
	10	6.5	0.51	0.16	1.3		4.8
	100	3.6	0.28	0.22	1.8	0.26	2.8

[a] Kinetic parameters were determined from analysis of Eadie-Hofstee and Webb plots

Figure 5


A:CIB7
B:AE-2
OBSERVED
THEORETICAL
PRETREATED WITH AE-2
CIB7 ALONE
PRETREATED WITH CIB7
AE-2 ALONE
INHIBITION OF AChE ACTIVITY (%)
100
80
60
40
20
0.1
1.0
10
100
MAb/AChE (molar ratio)